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Comparison of Lung Antioxidant Levels in Humans and Laboratory Animals

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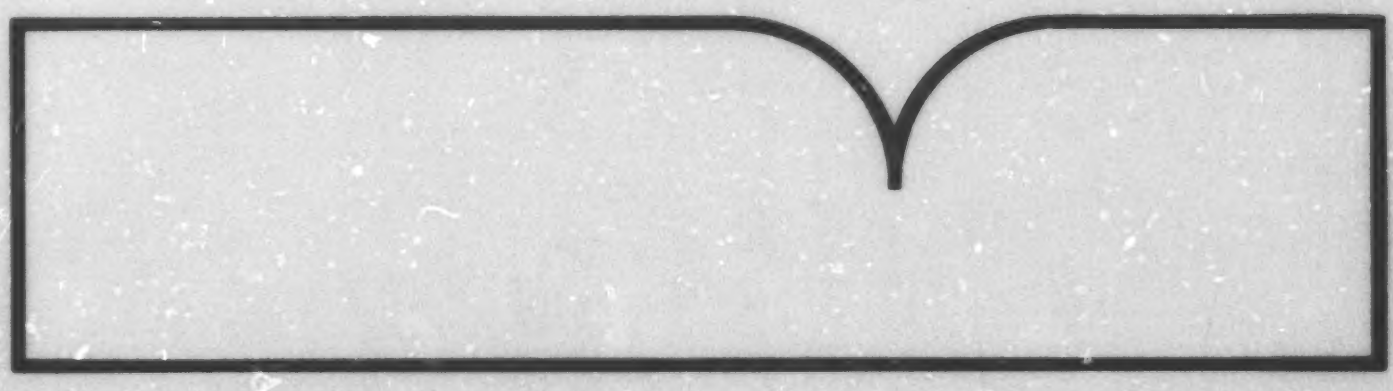

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16. ABSTRACT

Basal lung concentrations of ascorbic acid (AA), nonprotein sulfhydryls (NPSH), and a-tocopherol (a-T) were determined in rabbits, guinea pigs, rats, hamsters, mice, domestic pigs and sheep, and in human lung samples obtained from cancer surgery patients. Significant differences were found among the laboratory animals: AA levels ranged from 25.6 mg% (mg/100 g wet wt) in hamsters to 41.7 mg% in mice, NPSH from 50.9 mg% in rats to 84.7 mg% in rabbits, and a-T from 1.01 mg% in hamsters to 2.74 mg% in rats. Porcine lung AA and NPSH approximated those of the laboratory animals and their a-T levels were the highest of all species studied. Sheep lung AA levels were comparable to the other species, although their NPSH and a-T levels appeared to be lower. Human lung concentrations of NPSH (5.2 mg%) and a-T (0.85 mg%) appeared low, and the mean AA concentration (22.1 mg%) was similar to those of laboratory animals. A partial correlation was found between AA and a-T levels and between NPSH and a-T levels in the five laboratory animal species. The differences seen in the levels of these antioxidants may be indicative of differences in tissue sensitivity to oxidant-induced damage.

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JOURNAL ARTICLE

Comparison of Lung Antioxidant Levels in Humans and Laboratory Animals[1-3]

RALPH SLADE, ANDREW G. STEAD, JUDITH A. GRAHAM, and GARY E. HATCH

SUMMARY Basal lung concentrations of ascorbic acid (AA), nonprotein sulfhydryls (NPSH), and α-tocopherol (α-T) were determined in rabbits, guinea pigs, rats, hamsters, mice, domestic pigs and sheep, and in human lung samples obtained from cancer surgery patients. Significant differences were found among the laboratory animals: AA levels ranged from 25.8 mg% (mg/100 g wet weight) in hamsters to 41.7 mg% in mice, NPSH from 50.9 mg% in rats to 84.7 mg% in rabbits, and α-T from 1.01 mg% in hamsters to 2.74 mg% in rats. Porcine lung AA and NPSH concentrations approximated those of the laboratory animals and their α-T levels were the highest of all species studied. Sheep lung AA levels were comparable to those in the other species, although their NPSH and α-T levels appeared to be lower. Human lung concentrations of NPSH (5.2 mg%) and α-T (0.85 mg%) appeared low, whereas the mean AA concentration (22.1 mg%) was similar to that of laboratory animals. A partial correlation was found between AA and α-T levels and between NPSH and α-T levels in the 5 laboratory animal species.

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No. 5 (May)

Introduction

Intraspecies extrapolation of the toxicity of inhaled gases such as ozone and nitrogen dioxide must take into account differences both in dose delivered to the tissues and in tissue biochemistry. Mathematical modeling techniques are being developed for estimating species differences in the tissue dose of these gases at different levels in the lungs and airways (1). One approach to determining biochemical differences is through analysis of the components capable of protecting lung tissue from oxidant damage. These include antioxidant molecules such as α-tocopherol (α-T), nonprotein sulfhydryls (NPSH), and ascorbic acid (AA) as well as enzymes presumed to be involved in antioxidant defense (e.g., superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase).

Ascorbic acid, NPSH (of which glutathione is a major constituent), and α-T are known to be present in lung tissue at concentrations that should contribute significantly to the protection of the lungs from oxidant-induced damage (2-6). Under acute exposure conditions, direct reaction of an oxidant gas with these molecules may be an important defense, and species differences in their levels could indicate possible differences in tissue sensitivity.

Widely divergent values for AA (7,8), NPSH (4, 9), and α-T (10, 11) lung levels have been previously reported, probably because of differences in the methods of analysis used. There have been no studies that examined several species in which the levels of all 3 of these antioxidants were measured in the same lung. For this reason we decided to carry out a study in which the tissue levels of these 3 antioxidants were determined in the same tissue samples from humans as well as from several other species under the same analytical conditions, the results of which are described in this report.

Methods

Source of Animal and Human Tissues

All animals used in this study were young adults. Swiss albino mice (CRL: CD-1) weighing 20 to 30 g, Hartley strain guinea pigs, weighing 300 to 400 g, and Sprague-Dawley rats (CD), weighing 200 to 300 g, were obtained from Charles River Breeding Labs, Kingston, NY. New Zealand white rabbits, weighing 3,000 to 4,000 g, were purchased from Hazelton/Dutchland Laboratories Inc., Denver, PA, and Golden Syrian hamsters, weighing 100 to 150 g, were obtained from Engle Laboratory Animals, Farmersberg, IN. Animals were housed in temperature- and humidity-controlled rooms and fed standard diets obtained from Continental Grain Co., Chicago, IL or Ralston Purina Co., St. Louis, MO (rats). The α-T contents of the diets ranged from 35 to 66 ppm, and the guinea pig diet contained 1,000 ppm AA. Sheep (females weighing approximately 90 kg) and pigs (females weighing approximately 115 kg) were obtained locally.

Human lung tissue was kindly provided by Dr. Richard C. Boucher, M.D., of the Division of Pulmonary Disease, University of North Carolina School of Medicine, Chapel Hill. These samples were obtained from cancer surgery patients and were taken from apparently normal regions of the lungs. Pulmonary arterial and bronchial tissues were dissected out and assayed separately in some of the samples. Patients included both males and females of various ages and smoking histories.

Preparation of Tissue Samples

Laboratory animals (except rabbits) were anesthetized by a 1- to 2-min exposure to 5% halothane (Halocarbon Laboratories, Hackensack, NJ) using a Frazer-Sweatman vaporizing anesthesia apparatus (Cyprane North America, Tonawanda, NY). Rabbits were anesthetized by injection of pentobarbital into the marginal ear vein. Blood samples were taken by cardiac puncture, and 0.1-ml aliquots were immediately added to 3.0 ml metaphosphoic acid (on ice) and vortexed. The animals were exsanguinated by severing the abdominal aorta, and lung and liver tissue samples were quickly removed, weighed, and homogenized using a Polytron tissue homogenizer (Brinkman Instrument Co., Westbury, NY), equipped with an anaerobic probe generator. Lung tissue samples from humans were removed and placed in cold saline for 30 min, then stored at −70° C until assayed. Samples (approximately 1.0 g) of sheep and pig lung, which had been taken approximately 1 h postmortem, were immediately placed in liquid N_2 and stored there until used.

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[2] This report has been reviewed by the Health Effects Research Laboratory, U.S. Environmental Protection Agency, and approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

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Analysis of Tissue Ascorbic Acid and NPSH

Lung and liver samples (0.1 to 0.2 g) were taken at random from different parts of the lung for AA and NPSH analysis and were homogenized in 3.0 ml of 6.0% (wt/vol) metaphosphoric acid. The homogenates were centrifuged at 20,000 x *g* for 20 min at 4° C. Supernatants were used directly for analysis of NPSH by the method of Sedlak and Lindsay (12).

Homogenate supernatants were also used to assay for AA by the reaction of dehydroascorbic acid with dinitrophenylhydrazine (DNPH) (13). Approximately 1.0 ml of the supernatant was oxidized with Br_2 gas. The Br_2 was removed from the sample using water-saturated N_2, and 0.6 ml of the oxidized supernatant was added to 0.2 ml of 2.0% (wt/vol) DNPH and 0.25% (wt/vol) thiourea in 9.0 N H_2SO_4. The tubes were covered and allowed to sit at 25° C for 24 h (14); 1.0 ml of ice-cold 65% (vol/vol) H_2SO_4 was added, mixed, allowed to sit at 25° C for 30 min, and the absorbance at 525 nm was determined.

Analysis of Lung α-Tocopherol

Tissue samples (approximately 0.5 g) were homogenized in 10.0 ml of ethanol plus 2.5 ml of 25% (wt/vol) ascorbic acid. After homogenization, the samples were saponified by addition of 1.0 ml of 10.0 N KOH, flushed with N_2 for 1 min, and incubated under N_2 at 45° C for 20 min. The samples were placed on ice until cold, and 10.0 ml of cold hexane containing 1.25 mg% butylated hydroxytoluene were added. The samples were vortexed for 1 min, then centrifuged at 500 x *g* for 1 min. The hexane layers were removed and dried under N_2, and the residue was redissolved in 0.5 ml of methanol. Analysis of α-T was by the high-performance liquid chromatography (HPLC) method of DeLeenheer and coworkers (15). This method separates the α-T from other potentially interfering substances, such as retinol. Saponification of the samples was found to be necessary, since during HPLC analysis unsaponified samples gave numerous large peaks that interfered with the α-T peak and shortened the usable life of the C-18 column. Experiments using an internal α-T standard showed that these extraction and chromatographic techniques resulted in a 90 to 95% recovery of α-T from lung tissue.

Statistical Methods

For the 5 species of laboratory animals, analysis of variance was employed to test the influence of species and sex, as well as their interaction on lung antioxidant levels. Log transformation was used to achieve homogeneity of variances. For each antioxidant, means from each of the 5 species were compared using the procedure of Tukey (16) and Kramer (17). Although this procedure is more conservative than others are, it more effectively takes into account the probability of making at least one false positive statement over all possible comparisons of 2 means. Multivariate analysis of variance (18) was used to estimate the partial correlation between the 3 antioxidants, i.e., the relationship between these levels after adjusting for species differences. The overall correlation (no adjustment for species) was performed using the Pearson product-moment correlation.

The means and standard errors of lung antioxidant levels in humans, pigs, and sheep have been displayed along with the data from the laboratory animals. However, because of differences in sample sizes, health status, sex, age, and methods used to obtain and store tissue samples, the data from these 3 species were not included in the statistical analysis with the laboratory animal species.

Results

Human Tissue Antioxidant Levels

The data from the human tissue samples are presented in table 1. The AA levels from human lung parenchyma ranged from 1.95 to 66.4 mg%, with a mean value of 22.1 mg%, whereas the NPSH levels varied from 2.19 to 11.1 mg%, and the α-T levels ranged from 0.48 to 1.88 mg%, with a mean of 0.85 mg%. The bronchial tissues showed these same ranges of values. However, the mean bronchial levels of α-T (1.63 mg%) and NPSH (14.6 mg%) appeared to be higher than those of the parenchyma. The mean bronchial AA level was 22.3 mg%. The levels of these antioxidants were also determined in 6 samples of human pulmonary artery. The mean values for AA (16.4 mg%), NPSH (10.5 mg%), and α-T (0.89 mg%) in this tissue were roughly comparable to those of other pulmonary tissues. No apparent differences in lung antioxidant levels could be discerned from the data with regard to smoking status, sex, or age of the patients studied. For comparative

TABLE 1

ANTIOXIDANT LEVELS IN HUMAN PULMONARY TISSUE

Patient No.	Age (yr)	Sex	Smoker	Lung Parenchymal Tissue			Bronchial Tissue		
				Ascorbate†	NPSH†	α-tocopherol†	Ascorbate†	NPSH†	α-tocopherol†
1	61	M	+	–	6.00	0.88	–	–	–
2	71	F	–	–	2.19	1.88	–	–	–
3	*	*	*	–	3.27	0.81	–	–	–
4	*	*	*	–	4.25	1.12	–	–	–
5	56	M	+	–	–	–	5.13	19.77	1.30
6	55	F	–	–	–	–	10.11	4.21	0.68
7	60	M	+	66.41	3.27	0.66	40.90	6.89	1.14
8	52	M	+	–	–	–	33.99	34.46	1.08
9	46	F	–	43.27	4.97	0.57	17.05	7.80	0.31
10	21	M	–	26.20	6.45	0.55	–	–	–
11	56	M	+	–	–	–	62.35	5.30	–
12	60	M	+	16.50	3.76	1.29	–	–	–
13	64	M	+	30.23	4.55	0.63	–	–	–
14	*	*	*	12.27	4.20	0.82	–	–	–
15	*	*	*	2.90	7.01	–	2.91	6.73	0.58
16	51	M	+	–	–	–	19.16	10.02	2.41
17	72	M	+	15.62	11.07	0.51	–	–	–
18	*	*	*	–	–	–	7.23	36.11	5.52
19	76	F	+	3.95	5.20	–	–	–	–
20	56	M	+	1.95	6.97	0.48	–	–	–
Mean ± SE				22.13 ± 6.45	5.23 ± 0.59	0.85 ± 0.12	22.31 ± 6.89	14.59 ± 4.19	1.63 ± 0.60

* Data not available (blank spaces indicate insufficient tissue for assay).
† mg/100 g wet tissue (mg%).

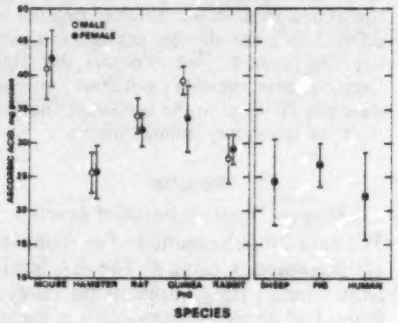

Fig. 1. Species comparison of lung AA concentrations expressed in mg/100 g wet tissue. Means and standard errors are shown for males and females separately in the laboratory animals. Numbers of animals/patients used were: 3 (sheep), 10 (pigs, humans), 6 (each sex in mice), 4 (each sex in remaining species).

purposes, means and SE from the human data are included with the animal data in figures 1 to 3.

Lung Antioxidant Levels in Domestic Sheep and Pig

Mean lung AA levels were 24.2 ± 6.4 mg% in sheep and 26.7 ± 3.2 mg% in pigs (figure 1). The pig lung NPSH value was 36.8 ± 1.9 mg%. In sheep anesthetized for 1 h with pentobarbital, the mean NPSH level was 23.9 ± 3.3 mg%, whereas in another group of sheep that were killed by stunning and exsanguination and from which the lung samples were immediately removed and placed in liquid N_2, the value was 56.1 ± 4.1 mg% (figure 2). The average level of α-T in the pigs was 3.55 ± 0.25 mg% and in the sheep was 0.66 ± 0.15 mg% (figure 3).

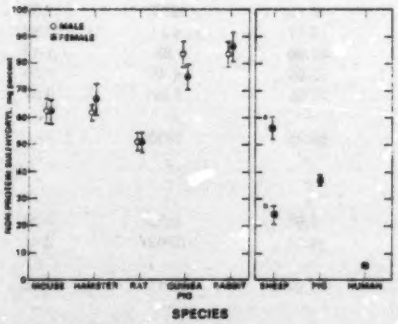

Fig. 2. Species comparison of lung NPSH concentrations expressed in mg/100 g wet tissue. Means and standard errors are shown for males and females separately in the laboratory animals. Number of animals/patients used were: 3 (each group of sheep), 10 (pigs), 14 (humans), 3 (each sex in laboratory animals). a. Killed by stunning and exsanguination. b. Anesthetized with pentobarbital.

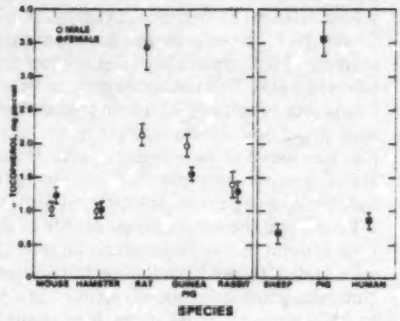

Fig. 3. Species comparison of lung α-T concentrations expressed in mg/100 g wet tissue. Means and standard errors are shown for males and females separately in the laboratory animals. Numbers of animals/patients used were: 3 (sheep), 10 (pigs), 12 (humans), 8 (each sex in laboratory animals).

Effect of Storage and Anesthesia on Lung NPSH Levels

Because of the low levels of NPSH in humans, sheep, and pigs, we tested the effects of storage of lung tissue at −70° C and of anesthesia on the lung NPSH levels in laboratory animals. In a sample of guinea pig lung tissue, the NPSH level of the fresh tissue was 75.9 mg%, after 1 wk at −70° C it was 73.6 mg%, and after 8 wk at −70° C it was 78.7 mg%. In a group of rats, anesthesia for 1 h with 2.5% halothane yielded rat lung NPSH levels of 60.2 ± 2.5 mg% (n=4) compared with those in control animals (5% halothane for 1 min) in which they were 53.5 ± 1.5 mg% (n=4). After 1 h of anesthesia with pentobarbital, the levels were 55.3 ± 2.5 mg% (n=4). Therefore, storage of tissue at −70° C did not appear to lower NPSH levels in guinea pig lungs, and anesthesia resulted in a decrease of less than 10% in rats.

Antioxidant Levels in Blood and Liver

For purposes of comparison and quality assessment of the assays, ascorbic acid levels were also determined in the blood and livers of the laboratory animals. There were no species or sex differences found in these levels from either blood or liver. The values for liver ranged from 17.6 mg% ± 3.7 SE (n=5) in rabbits to 26.8 mg% ± 8.0 SE (n=5) in hamsters. For blood AA levels, the values ranged from 0.56 mg% ± 0.11 SE (n=8) in rats to 0.87 mg% ± 0.41 SE (n=4) in mice.

Influence of Sex Differences on Lung Antioxidant Levels

Lung AA and NPSH levels did not vary significantly by sex in the 5 species of laboratory animals (figures 1 and 2). There was a significant difference ($p = 0.02$) in the lung α-T levels between male (2.08 mg%) and female (3.31 mg%) rats (figure 3). No sex differences in α-T were found in any of the other 4 species. Because of the sex difference found in rats, subsequent one-way ANOVA was done on each sex separately to compare levels across species. There were significant ($p < 0.01$) differences between species in mean α-T levels regardless of sex. The multiple comparison procedure applied to these levels in females indicated that rats had significantly higher levels than did all other species. In males, rats had significantly higher α-T levels than did all species except guinea pigs. In the species comparison studies discussed below, data from males and females have been combined for analysis.

Comparison of Lung Tissue Ascorbic Acid Levels in Laboratory Animals

In the 5 species of laboratory animals, the mean AA levels ranged from 25.6 mg% in hamsters to 41.7 mg% in mice (figure 1). These levels varied significantly according to species ($p < 0.01$): mice had higher lung AA levels than did rabbits and hamsters, and guinea pigs had levels higher than those in hamsters, according to the Tukey/Kramer statistical analysis.

Comparison of Lung Tissue NPSH Levels in Laboratory Animals

The lung nonprotein sulfhydryl levels ranged from 50.9 mg% in rats to 84.7 mg% in rabbits (figure 2). Lung levels of NPSH also varied significantly by species ($p < 0.01$): the multiple comparison procedure indicated that rabbit and guinea pig levels were not different from each other but were higher than levels in hamsters, mice, and rats; hamster and mouse levels did not differ significantly but were higher than rat lung levels.

Comparison of Lung Tissue α-T Levels in Laboratory Animals

Lung α-T mean levels among the 5 species of laboratory animals ranged from 1.01 mg% in hamsters to 2.74 mg% in rats (figure 3) and varied significantly by species ($p < 0.01$). Rats had higher levels than did the other 4 species, and guinea pigs had higher levels than mice, hamsters, and rabbits, among which there were no significant differences.

Comparison of Molar Concentrations of Lung Antioxidants in Laboratory Animals

On a molar basis, the antioxidants had the following range of concentrations: AA, 1.45 to 2.37 mM; NPSH, 1.66 to

TABLE 2

CORRELATION COEFFICIENTS BETWEEN ANTIOXIDANT LEVELS IN LUNGS

Correlation	AA α-T	NPSH α-T	NPSH AA
Overall			
r	−0.380	0.017	0.082
p value	0.067	0.939	0.703
Partial			
r	−0.585	0.494	−0.032
p value	0.007	0.027	0.895

2.75 mM; α-T, 0.024 to 0.065 mM. Comparison of these molar concentrations in each species revealed that AA is 29 to 88 times more abundant in whole lung than is α-T, and NPSH is present at levels that are 26 to 89 times greater than those of α-T. The range of NPSH to AA concentrations varied from approximately 0.9 in rats and mice to 1.3 to 1.7 in the other species.

Correlations between Lung Antioxidant Levels

Analysis of antioxidant levels in individual species indicated a significant negative correlation between AA and α-T levels in mice ($p = 0.002$, $n=8$) and rabbits ($p = 0.034$, $n=4$) but not in the other species. After adjusting for sex and species effects (partial correlation) and considering only those cases in which all 3 antioxidants were measured in lung tissue from the same animal ($n=24$), a significant ($p = 0.007$) negative correlation was found between AA and α-T levels in the 5 laboratory animal species (table 2). A significant ($p = 0.03$) positive correlation was seen between α-T and NPSH lung levels. The partial correlation between AA and NPSH levels was not significant. No significant correlations were found when the pooled data were analyzed without correcting for sex and species (overall correlation).

Discussion

In this study we have demonstrated that species differences exist in the lung levels of 3 antioxidants: α-tocopherol, nonprotein sulfhydryls, and ascorbic acid. These differences could be indicative of tissue sensitivity differences to oxidants and possibly other inhaled pollutants. The lung levels of AA that we observed in the laboratory animals fell within a range (lowest to highest) of less than 40%. This range of values is roughly comparable to those previously reported for rats (19), guinea pigs (20), and mice (21). However, some investigators have reported lung AA levels in guinea pigs that were substantially higher (65 mg%) (7) as well as lower (7 mg%) (8) than those that we observed. Stubbs and McKernan (19) reported a significant difference of 20% in the AA lung concentrations in male and female rats. We found no significant sex-related differences in AA levels between any of the species of laboratory animals.

The NPSH concentrations in the laboratory animals also had a range of about 40% across the 5 species. Previously published values for guinea pigs (7) and rats (4) are in agreement with our values. However, lung NPSH concentrations have been reported that were twice as high in rats (9) and 3 times as high in mice (21) as those seen in this study.

The values for lung α-T levels varied by 71%. Our observed levels are comparable to those reported previously for rats (10) and guinea pigs (7). On the other hand, other investigators have reported lower values in these same species (11, 20, 22). Lung α-T is obviously present at the lowest concentrations among the 3 antioxidants we have measured. On a molar basis its concentration in the lungs of the 5 species is only about 1 to 4% of the NPSH or AA levels.

The magnitude of these differences in the lung concentrations of AA, NPSH, and α-T is within the range obtained in laboratory animals using diet or drugs to lower antioxidant levels (6, 22–26). Although alterations of tissue antioxidant contents have been associated with increased susceptibility to oxidant-induced damage, it is uncertain whether interspecies variations in antioxidant levels can be used to predict species differences in oxidant sensitivity. We have recently compared the edema response of the same 5 species of laboratory animals used in this study induced by 0.2 to 2.0 ppm O_3 and found no apparent relationship between lung AA, NPSH, and α-T levels and edema among these species (report in preparation). Similarly, no definite relationship is apparent to us between the data presented here and numerous other species comparison studies of oxidant gas sensitivity reported in the literature (27–29). It appears that relating lung antioxidant levels to *in vivo* sensitivity may be difficult because biochemical mechanisms of antioxidant protection may vary considerably for different oxidants (O_3, NO_2, 100% O_2, etc.) and also because the dose of oxidant actually delivered to the lungs probably varies widely from species to species. We are presently addressing the question of delivered dose using gases labeled with oxygen-18. Further work is also required to identify the particular class of antioxidant that most effectively protects against each type of oxidant exposure.

The levels of NPSH and α-T in human tissues were particularly low relative to other species studied, whereas the AA levels were about the same as those seen in the laboratory animals. Human lung concentrations of AA as low as 7 mg%, which is about one third the mean lung level we found, have been previously reported (30). Bieri and Evarts (31) found α-T levels in human lung samples ranging from 0.39 to 1.68 mg%, which are within the range we observed. The low levels of NPSH and α-T in our study may have been due to the fact that all of the human lung samples were obtained from cancer patients. Although the samples were taken from apparently normal regions of the lungs, it is possible that the antioxidant levels in these regions were altered by the neoplasia or by chemotherapy, radiation therapy, or changes in diet. It is unlikely that methods of storage of the human lung tissues (−70° C in air for 2 wk to 1 yr) contributed significantly to these low levels, since storage of animal tissues at −70° C for as long as 8 wk did not seem to alter NPSH levels. The anesthesia used with the human subjects could also have affected the antioxidant levels, since NPSH levels were low in sheep that had been anesthetized for at least 1 h compared with those in unanesthetized sheep. However, our experiments with pentobarbital and halothane anesthesia in rats showed no evidence of a decline in the levels of NPSH. Further studies are needed with the larger animals.

It has been theorized that AA, NPSH, and α-T are interrelated in providing protection from *in vivo* oxidative tissue reactions. Ascorbic acid (32, 33) or NPSH

(34) working alone or in combination may be responsible for reducing α-T after its oxidation (35). Furthermore, changes induced in the levels of one or more of these antioxidants have been reported to affect the levels of the others (2, 8, 36). We found significant partial correlations between AA and α-T lung levels as well as between α-T and NPSH levels, suggesting that individual animals within a species are correlated. However, the lack of correlation observed when species effects are not corrected suggests that this pattern does not hold when comparing across species. These findings lend further support to the idea that the antioxidants are interrelated, although the mechanism behind the interrelationships is still not understood.

To summarize, this study was carried out to see if species differences could be demonstrated in the lung levels of 3 antioxidants, AA, NPSH, and α-T. We found that significant differences existed in these levels in 5 species of laboratory animals. Although significant partial correlations were found between AA and α-T levels and α-T and NPSH levels within individuals of these 5 species, no such correlation was observed when the data were compared across species. We also found that human lung AA levels were similar to those of the laboratory animals, whereas the human NPSH and α-T concentrations were lower. Ascorbate levels in domestic pigs and sheep and NPSH levels in pigs were about the same as the levels in laboratory animals, whereas NPSH and α-T levels in sheep were lower. Pigs had the highest α-T levels of all species studied.

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